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Mutation of the p53 gene is very frequent in breast cancer. In normal cells induction of wild-type p53 function leads to either cell cycle arrest or cell death. Loss of this function can contribute to oncogenic cell transformation. Additionally the presence of mutant forms of p53 in breast tumor cells may actually facilitate the process of tumorigenesis. The properties of mutant p53 proteins in vitro and in breast tumor cell lines will be studied, experiments will focus on analysis of the structure and modification of mutant p53 proteins as well as the effect of cellular signaling on p53 function. Additionally it is planned to establish breast cell lines expressing inducible mutant p53 to determine the effect of such mutants on parameters of cell cycle, growth and death.

We have discovered that all mutant forms of p53 tested are capable of binding specifically to p53 response elements present in p53 target genes at lower but not at physiological temperatures. Furthermore, we have identified a means by which such binding is stabilized at the higher temperature. This will allow us to explore means to develop molecules that might have the outcome of converting p53 in breast tumor cells from mutant to wild-type function. One approach will be to develop a yeast-based screen for mutant p53 modifying genes. Such reagents would have clear therapeutic advantages.

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Carol Reeves 1/21/99

PI - Signature Date

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DAMD-17-94-J-4275

The goal of the research funded by DAMD-17-94-J-4275 is to characterize the role of mutant p53 in breast cancer progression and to develop means to counteract the tumorigenic potential of mutant p53.

INTRODUCTION:

The p53 tumor suppressor protein plays a pivotal role in transmitting a signal from agents that induce genotoxic stress to genes that control the cell-cycle and apoptosis (1,2). p53 is a DNA binding dependent transcriptional activator which binds specifically to sites in genomic DNA that contain two or more copies of the consensus sequence: 5' R R R C A/T T/A G Y Y Y 3' (3). Such sites are identified as p53 response elements in a number of genes. Thus when cells are stressed by processes such as DNA damage or hypoxia the p53 protein normally present in low quantities in cells and in a latent, inert form, is activated both quantitatively and qualitatively to induce several target genes. Among these are included the genes expressing GADD45 (4), WAF1/p21/CIP1 (5), mdm2(6), cyclin G (7), bax (8) and IGFBP3 (9). Each of these genes contains a p53 response element and is therefore a likely target for p53 as a transcriptional activator to induce their expression. Each thus is likely to play a role in the p53 pathway in which, as a result of DNA damage, normal cells either undergo cell cycle arrest or cell death. When p53 is mutated and cannot respond thus to DNA damage, cells display the loss of growth control that is characteristic of tumorigenesis.

Mutation of the p53 tumor suppressor gene is among the most frequent events in breast cancer. Such mutation is frequently manifested as loss of one allele coupled with missense mutation of the other allele. Strikingly the location of the missense mutations are within the central region of the molecule (10) and this region contains the DNA binding domain (11). This highlights the likelihood that specific DNA binding and sequence specific transactivation is essential for the tumor suppressor function of p53 and that DNA binding is absent from mutant forms of p53. The missense mutant p53 proteins are frequently expressed at very high levels in breast tumor cells (12), and the p53 status in breast cancer has been linked closely to detection of p53 protein by immunostaining (13). Therefore understanding the function of the wild-type p53 protein and how it is altered when p53 is mutated will be critical to evaluating the prognosis of breast cancer. Importantly, the study of the properties of mutant p53 in breast cancer will hopefully lead to the development of ways to convert mutant protein to wild-type in function. The original aims of this proposal are as follows:

Specific Aims:

(1) Analysis of the structural properties of mutant forms of p53 that are found in breast cancer and how they differ from that of the wild-type form as well as among themselves.

(2) Analysis of the DNA binding properties of mutant forms of p53 in breast cell lines with the aim of (a) finding cellular genes that are targets of mutant p53 activation and (b) conversion to or stabilization of the wild-type DNA binding activity of mutant forms of p53.

(3) Identification of cellular proteins from mammary cell lines that might be involved in mutant p53 gain-of-function in breast cancer.

BODY

Work supported by **DAMD-17-94-J-4275** resulted in 6 publications in peer reviewed journals, and it is expected that at least two more papers will be submitted under the auspices of this grant

The work accomplished will be described as it pertains to the 3 tasks.

Progress made with respect to the Statement of Work

As I explained in my last report, much of what we set out to accomplish has changed due to discoveries made in ours' and others' laboratories. Overall, however, the goal of our grant is to study and modulate mutant p53 function in order to eventually develop p53-based therapeutics for treatment of breast cancer remains the same. Thus the description of the tasks and our progress therein is very similar to that of the last report. Where additional progress has been made, the text is italicized.

Task 1: Examination of the properties of mutant p53 proteins:

(a) biochemical analyses of mutant p53 immunopurified proteins expressed from currently available baculoviruses: phosphate mapping, proteolysis protocols, hsp binding and oligomerization analyses

As outlined in the progress report from last year, we have examined the ability of mutant p53 proteins to be phosphorylated by cyclin dependent kinase, have examined their state of oligomerization by sucrose gradient sedimentation analysis and non-denaturing gels. Neither of these tasks are ready for publication at present because the initial results did not provide any new insight into why mutants differ from wild-type protein with respect to DNA binding. With respect to examination of the effect of proteolysis, we did have some success. Much of our work, as mentioned in the original report was predicated on our discovery of the temperature sensitive phenotype of mutant p53 proteins and so we have tried to further understand this property in biochemical terms. We have discovered that wild-type p53 is also temperature sensitive such that it fails to resolve on non-denaturing gels at 37°C in contrast to experiments performed at 25°C. We observed that creatine phosphate will stabilize wild-type p53 at 37°C both for DNA binding and gel resolution. However, creatine phosphate does not stabilize mutant p53 at 37°C.

(b) Cultured breast cell lines: develop purification procedures for mutant p53 from cells.

Since the last report we have focussed on examination of the DNA binding properties of p53 from extracts of yeast and mammalian cells. We have established conditions whereby p53 DNA binding can be detected in the absence of the "activating antibody" PAb 421.

(c) comparative analyses of breast cell derived p53 proteins.

We did not develop procedures for the isolation of mutant p53 from breast cell lines because our preliminary experiments did not warrant this at the time. Thus, having not documented any significant differences with purified proteins it did not seem effective to proceed with this line of research.

(d) construction of baculoviruses expressing additional mutant p53s found in breast cells with aim of continuing comparative analysis of different mutant forms of p53..

When we started our research on mutant p53 supported by DAMD17-94-J-4275 we had generated baculoviruses expressing four of the mutant forms of p53 as baculovirus expression vectors. Since then we have successfully constructed a fifth, ser 149, and are in the process of making the sixth (and final) "hotspot" mutant, ie mutated at codon 282. We have actually had a number of cloning difficulties with this, but are optimistic that we will solve our problem. Still no progress at the present time has been made in generating this additional baculovirus. However, we have initiated a project in which p53 is expressed in and purified from bacteria and are hopeful that this will provide an excellent and abundant source of wild-type and mutant p53 protein. During the final stage of this grant support period it is planned to examine many of the properties outlined in Task 1 using this source of p53 protein.

Task 2: Analysis of the DNA binding properties of mutant p53 proteins:

(a) Continue studies on the temperature sensitive phenotype of mutant p53 proteins performing gel-shift, DNase I footprinting and methylation interference assays. Examine exiting p53 response elements, adding more as they become available from the literature. As new mutants become available, determine the generality of the ts phenotype.

(1) The role of the N-terminus in regulation of DNA binding of wild-type and mutant forms of p53.

We previously identified a region within the N-terminus of p53 approximately encompassing residues 45-55 that when bound by antibody will stabilize mutant p53 binding at 37°C (14). To extend this finding we have further characterized the role of the N-terminus in regulating DNA binding by the central core domain of p53: We found that an antibody which binds to the N-terminus (PAb 1801), which recognizes residues 46-55, affects the rate of disassociation of p53 from DNA. We have acquired additional N-

terminal-specific antibodies and have tested them for their ability to stabilize wild-type p53 DNA binding. Quantitative competitive gel mobility shift assays and DNaseI footprinting have been used to reach these conclusions. This project is well underway and it is expected that we will be able to submit a manuscript within a few months.

(2) We went on to examine the temperature sensitive phenotype *in vivo*. However, the results mandated a more careful analysis of the effects of transfected mutant p53 in tumor cells and these data yielded the interesting and important observation that one mutant, ala143, is defective in inducing apoptosis at 32°C in contrast to wild-type p53, but discriminates between p53 responsive genes. This was a collaborative study with M. Oren (Weizmann Institute) and was also recently published (15).

(b) Search for consensus sites for mutant p53 proteins operating selection assays at both 25°C and 37°C. Once identified, use sites as response elements in reporter assays in insect cells (grown at 27°C) or mammalian cells at 37°C.

A graduate student, T. Zhang, spent one year trying to isolate by using an established PCR based selection protocol DNA sequences that are bound by mutant forms of p53. She was unable to get the protocol to work, and, had to move onto other studies. We will, however, continue to explore this possibility given our exciting observation that some mutant p53 proteins display altered promoter specificity.

(c) Expand observations that PAb 1801 stabilizes DNA binding by mutant proteins: (i) generate mutants within the 1801 epitope region (ii) expression of N-terminal region of p53 with the aim of generating additional antibodies to further characterize the stabilization effect (iii) isolation of peptides from a phage display library that bind to the 1801 epitope region of p53 and testing whether they can stabilize mutant p53 DNA binding at 37°C.

We have recently initiated experiments designed to characterize the PAb 1801 epitope. As described above we have made the very interesting observation that, in contrast to antibodies directed against other epitopes in p53, this antibody has a marked effect on the dissociation rate constant of p53 bound to its cognate DNA. We attempted repeatedly to generate a baculovirus expressing the N-terminus of p53 without success. This may be due to the observation that the N-terminus has very little structure and is very protease sensitive. However, we have made a construct expressing the N-terminus fused to GST and can cleave the N-terminus (amino acids 1-83) from the GST. We will attempt to generate antibodies to this N-terminal peptide. Extensive mutagenesis of the 1801 epitope region is also planned.

Task 3. Identification of proteins or peptides that interact with mutant p53.

Attempts to identify proteins that interact with mutant p53 are ongoing. In addition to using columns and immunoprecipitations from tumor cell lines, we have initiated a yeast screening program to identify factors that interact *functionally* with mutant p53 to restore its transcription function which is our penultimate goal. Establishing this system has led to two new papers from our group which were published in the past year. Even though

these experiments do not conform strictly to what we originally planned, they certainly conform in the best spirit of our intended goal which is to identify ways to convert mutant forms of p53 to the wild-type form in function. While we intend to pursue the other goals in parallel, i.e. identifying small peptides that can function in a similar matter, we need to find another collaborator or source of phage display libraries since our original collaborator, C. Siegal, is no longer working in this area. On a related note however, we had tried the effects of two peptides that had been isolated by screening a phage display library with our wild-type and mutant p53 proteins, and failed to see any effect of these peptides on either wild-type and mutant forms of p53 protein. We have been offered assistance by a colleague with experience in this area, and if necessary we will purchase a phage display library for these experiments. It should be noted that we have made what I think is an important discovery regarding p53 in 1996, namely that there are a class of promoters that are activated by wild-type but not by mutant, apoptosis-defective forms of p53 (15). Furthermore, our data in yeast imply that the situation is far more complicated. Based on these observations, the original aim was modified such that we ascertain which sites that mutants can bind to are relevant to induction of apoptosis. Thus, one good bet would be to require that a mutant be converted to a form that can bind to and activate the bax promoter. Although it is not clear whether ba induction is necessary and sufficient for apoptosis in breast cancer cells, this would still give an idea if we could convert mutant p53 to the most functional version of wild-type p53 as well.

Since submitting the most recent progress report (12/08/97) there ha ve been two new publications funded by DAMD 17-94-J-4275. These involve the use of our yeast based assay to study the function of tumor derived mutant forms of p53. Additional progress was also made which will hopefully be submitted for publication with respect to the statement of work of the original proposal.

Abstracts of the two new publications are:

Human tumor-derived p53 proteins exhibit binding site selectivity and temperature sensitivity for transactivation in a yeast-based assay. *1998. DiComo, C.J. and C. Prives. Oncogene 16: 2527-2539.*

Abstract:

p53 is a sequence-specific transcriptional activator with a number of known target genes which contain p53-responsive elements. Mutations in p53 have been identified within its sequence-specific DNA binding domain in more than half of all human tumors, although a subset of tumor-derived p53 mutants have retained the ability to bind DNA and activate transcription under certain conditions. In order to broaden our understanding of this transactivating ability, we examined the efficacy by which p53 mutants bind to and activate reporters in an Saccharomyces cerevisiae-based assay. Analysis of nineteen human tumor-derived p53 mutants, spanning the DNA binding domain of p53 and including

the "hot-spot" class, revealed a broad array of transcriptional transactivation abilities at 24 °C, 30 °C, and 37 °C, despite the fact that each mutant had originally been identified as being inactive for transactivation in yeast against a single p53-responsive RGC site-containing reporter. One class of mutants (P177L, R267W, C277Y, and R283H) retained wild-type or near wild-type activity that is binding site-selective, even at physiological temperature (37 °C). Another class of mutants (V143A, M160I/A161T, H193R, Y220C, and I254F), all positioned for maintaining the β-scaffold of p53, also retained selective activity, but preferentially at sub-physiological temperatures (24 ° and 30 °C). Strikingly, however, in contrast to the other tumor derived mutants, all of the previously identified "hot-spot" mutants were completely inactive with all sites tested. Moreover, a double mutant, L22E/W23S, located within the activation region and previously shown to be transcriptionally inactive in fibroblasts, retained wild-type or near wild-type binding site-selective activity in yeast. Finally, we found that transcriptional activity in vivo does not necessarily correlate with DNA binding in vitro.

P73 Function is Inhibited by Tumor-Derived p53 Mutants in Mammalian Cells
Dicomo, C.J., Gaiddon, C. and C. Prives. 1999. Mol. Cell. Biol. In press.

Abstract: The p53 tumor suppressor protein, found mutated in over 50% of all human tumors, is a sequence-specific transcriptional activator. Recent studies have identified a p53 relative, termed p73. We were interested in determining the relative abilities of wild-type and mutant forms of p53 and p73α and β isoforms to transactivate various p53 responsive promoters. We show that both p73α and p73β activate the transcription of reporters containing a number of p53-responsive promoters in the p53 null cell line H1299. However, a number of significant differences were observed between p53 and p73, and even between p73α and p73β. Additionally, an S. cerevisiae-based reporter assay revealed a broad array of transcriptional transactivation abilities by both p73 isoforms at 30 °C and 37 °C. Recent data has shown that p73 can associate with p53 by yeast two-hybrid. We examined complex formation in transfected mammalian cells and found that p73α co-precipitated with mutant, but not wild-type, p53. Since many tumor-derived p53 mutants are capable of inhibiting transactivation by wild-type p53, we tested the effects of two representative "hot-spot" mutants (R175H and R248W) on p73. By co-transfecting p73α along with either p53 mutant and a p53-responsive reporter, we found that both R175H and R248W reduced the transcriptional activity of p73α. This decrease in transcriptional activity is correlated with the reduced ability of p73α to promote apoptosis in the presence of tumor-derived p53 mutants. Our data suggest the possibility that in some tumor cells an outcome of the expression of mutant p53 protein might be to interfere with the endogenous p73 protein.

Future Directions:

(1) Screening for gene products which can convert mutant p53 to wild-type in function.

Using the yeast-based assay we have completed the first phase of a project in which we will screen for genes that can enable mutant p53 to activate transcription from wild-type p53 responsive promoters (16). Select strains will be transformed with either a human or mouse cDNA expression library (*URA3* marked) which has been constructed from a cell line where the status of p53 is known. Preferentially, p53 should be either mutated or deleted to reduce the amount of false positives obtained when screening (wild-type p53 would bind to and activate the reporter). Additionally, we will use a high copy number yeast genomic library (*URA3* marked). Budding yeast may contain a protein(s) capable of binding to and restoring wild-type function to mutant p53. Regardless of the library used, transformants will be replica plated onto His^- medium and scored for those colonies which grow at 30C, as well as 37C. The assumption is that only those library plasmids which encode for a protein(s) which can interact with or modify mutant p53 and confer wild-type DNA-binding function will grow in the absence of histidine. However, the possibility exists that the library plasmid may encode a protein which binds to and activates the reporter construct on its own. To test this, all library plasmids isolated will be retransformed back into the reporter strain and assayed for their ability to grow on His^- medium. We are interested only in those library plasmids which require mutant p53 to grow on His^- medium. Additionally, the library plasmids isolated will be assayed for the ability to allow growth on His^- medium when other tumor derived mutants are present in the assay (e.g.: $p53^{R175H}$, $p53^{R248W}$, and $p53^{Y220C}$). It is important to show that the library plasmid is not specific for one tumor derived mutant but for a family of tumor derived mutants. Based on the results of the initial analysis described above, should genes be identified that confer wild-type activity on mutants it will be necessary to examine a number of different p53 response elements in order to determine the effectiveness of such gain-of function modifying genes.

(2) Use of conditional cell lines to identify cellular proteins with interact with mutant p53.

As described in previous progress reports and in Chen et al. (17) we have established a number of human tumor cell lines which contain inducible p53 genes. Most of the work since the last report has utilized H1299 cells containing either wild-type p53, tumor derived mutants (his175, ser249, trp248), p53 (22/23), p53Δ30 (amino acids 1-363) and p53 Δ62-91 (amino acids 1-61 fused to 92-393). One question we have posed addresses whether wild-type p53 can cooperate with anti-neoplastic agents to induce cell death and if so, which aspect of p53 is involved in this cooperation. We have observed that tumor derived mutants are incapable of inducing apoptosis, as measured by quantitation of cells with sub-G1 content of DNA, or by Annexin staining, under any conditions tested to date. However, wild-type p53 cooperates synergistically to induce cell death by the same experimental criteria, with a number of anti-neoplastic agents, including

etoposide, daunorubicin, 5-fluoro uracil, camptothecin and hydroxyurea. p53,however, fails to cooperate with the spindle poison colchicine. Importantly, by Western blot analysis there are no significant changes in the level of p53 protein detected before or after treatment with any of the above agents. It is planned to use these lines to identify proteins which may interact with mutant forms of p53 when induced.

It is planned as well to use these lines to determine whether there may be proteins which can interact with mutant forms of p53 after induction. Cells will be maintained in the presence of tetracycline. Cells will be labeled with [^{35}S] methionine for two hours prior to extraction, approximately 12 hours after withdrawal from tetracycline. Cell extracts will be made and subjected to sucrose gradient centrifugation in order to obtain a partial fractionation of p53 protein complexes present in cells. Immunoprecipitates of p53 proteins in gradient fractions will be resolved by SDS PAGE. A comparison of wild-type and mutant forms of p53 will be made. Proteins specifically immunoprecipitated with either form of p53 will be further studied and eventually identified by microsequencing methods.

CONCLUSIONS:

The work sponsored by this grant led to a number of conclusions which are briefly summarized as follows:

(1) Many mutant forms of p53 including ones that are commonly found in breast concer cells are temperature sensitive for binding to a number of DNA sites found in p53 responsive genes.
(2) Monoclonal antibodies directed against epitopes in the N-terminus of p53 are capable of stabilizing temperature sensitive binding by p53 mutants.
(3) Wild-type p53 is also temperature sensitive for DNA binding, although significantly less so that tumor-derived mutant forms of p53. The rate of disassociation of wild-type p53 from DNA is slowed markedly by incubation with N-terminal antibodies, suggesting a mechanism for the effect of these antibodies on thermal sensitivity of the protein.
(4) Mutant forms of p53 are also capable of transactivating some p53 targets in cells maintained at reduced temperatures. One temperature sensitive mutant, ala143, is capable of activating a number of p53 responsive targets in cells at 33 C. However, it is incapable of inducing apoptosis. Since its ability to activate p53 responsive targets is restricted (it cannot induce transcription from the bax or IGF-BP3 promoters, in contrast to wild-type p53), this suggests a possible explanation for its defect in inducing apoptosis. This further suggests that p53 gene regulation may be more complex than previously assumed, and that there may be one or more classes of genes in cells which are differentially regulated. Thus, the original proposal to search for small molecules which are capable of activating the DNA binding properties of mutant forms of p53 was considered to be over-simplified.
(5) Based on the above conclusion, it was decided to establish a yeast-based assay as a more informative means to select for proteins/activities which can restore activity to mutant forms of p53. We discovered that in this assay, when a number of p53 mutants

were examined to activate transcription in yeast, only the "hot-spot" mutations which are greatly over-represented in human cancers, including mammary tumors, were completely defective in activating a large selection of p53 target genes. More recently, upon the discovery of p53 family members, we decided to investigate the possibility that mutant forms of p53 can interact with p73 proteins. We discovered that two mutant p53 proteins, R248S and R175H, are capable of binding to and down-regulating the transactivation and apoptotic functions of p73α. These data suggest the possibility that in tumor cells expressing p53 family members, the putative pro-oncogenic function of tumor derived mutant p53 proteins may be the result of down-regulation of other p53 family members.

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Meeting abstracts

Meeting: Frederick Hood College Tumor Suppressor and Cancer Genetics Meeting, June 3-8 1995.
Title: Stabilization of Conditional DNA Binding by Mutant p53 Proteins
Authors: Friedlander, P., Y. Legros[1], T. Soussi[1] and C. Prives.
Affiliations: Department of Biological Sciences, Columbia University, N.Y. 10027 and [1]Institut de Genetique Moleculaire, I.N.S.E.R.M., Paris.

Meeting: US Army DoD Breast Cancer Research Program. October 31-Nov 4 1997
Title: Mutant p53 function in vitro and in vivo.
Authors: C. Prives*, X. Chen, C. DiComo, P. Friedlander, T. Zhang,
Affiliation: Department of Biological Sciences, Columbia University, N.Y. N.Y., 10027

Meeting: Cold Spring Harbor Cancer Genetics & tumor Suppressor Genes, August 18-22 1996
Title: p53 levels, functional domains and DNA damage determine the extent of the apoptotic response of tumor cells.
Authors: Xinbin Chen, Linda J. Ko, Lata Jayaraman and Carol Prives
Affiliation: Department of Bioloigcal Sciences, Columbia University, N.Y. 10027

Meeting: Cold Spring Harbor Cancer Genetics and Tumor Suppressor Genes, August 19-23 1998.
Title: P53 mutants exert a dominant inhibitory effect on p73 function.
Authors: Charles DiComo, Christian Gaiddon and Carol Prives
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List of personnel who were paid by DAMD-17-94-J-4275

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